XXXIV. ANAPHYLAXIS TO THE SEPARATED PROTEINS OF HORSE-SERUM.

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The earliest attempt to examine the part played by the different serum proteins in anaphylaxis to a foreign serum was made by Gay and Adler [1908]. Gay and Southard had based a theory of the anaphylactic condition on an erroneous interpretation of the phenomenon of passive anaphylaxis. This theory explained the sensitive condition as due to a remnant of the foreign serum, introduced at the sensitising injection, and still present in the system. Clearly it might be expected that the remainder thus conferring sensitiveness would belong to a different fraction of the serum protein from that which, at the second injection, acted as a poison. In accordance with this expectation, Gay and Adler found that the euglobulin fraction of a serum had great power of producing sensitiveness to the serum from which it was separated, but no toxic action when reinjected into the sensitised animal. Taking further fractions they observed a progressive decline of sensitising power, and increase of toxicity to the sensitised animal, as the proportion of ammonium sulphate needed for precipitation became greater; so that the highest fraction, the albumin, had practically no power of producing sensitiveness, but a maximum toxicity for animals sensitised with the whole serum, or with the lower fractions. These conclusions are in complete disaccord with the later results, obtained by various investigators, on anaphylaxis to pure proteins, of animal or vegetable origin. Such results have indicated a considerable degree of specificity of the reaction, not only for the species from which the protein originated, but even for pure proteins separated from an organ or tissue.

Doerr and Russ [1909, 1], repeating Gay and Adler's experiments with

more attention to quantitative detail, obtained an entirely different result. Like Gay and Adler, Doerr and Russ worked with guinea-pigs, and separated the proteins of the ox or horse-serum used for sensitising by fractional precipitation with ammonium sulphate. They found, however, that sensitising power, and toxicity for the sensitised animal, declined in a parallel manner, with increase of the proportion of ammonium sulphate necessary for precipitation; so that the euglobulin fraction exhibited the maximum anaphylactic activity in either direction, the albumin being, in both respects, practically inert, and the pseudoglobulin occupying an intermediate position. Doerr and Russ's results are in conformity with the general tendency of the evidence obtained with other proteins. The only anomaly is the failure of the albumin fraction to act as an anaphylactic antigen. In a later paper [1909, 2], however, they showed a parallel failure of serum-albumin to produce a precipitin when injected into the rabbit, and, in conformity with their view of the identity of anaphylactic antibody with precipitin, a failure of serum from a rabbit so injected to confer passive anaphylaxis on the guinea-pig.

This question of the antigenic properties of the separate serum proteins, with regard to precipitin formation, has been examined by several other investigators, with results which show the widest possible variation, both in respect of the relative efficacy shown by the different proteins in exciting precipitin formation, and of the specificity to the individual proteins of the precipitins obtained. Some agree with Doerr and Russ as to the absence of a precipitin reaction to serum albumin; others, while obtaining a precipitin by injection of albumin, find that it precipitates even more strongly with globulin than with the albumin exciting its production. Doubtless a good deal of the variation is due to the varying degrees, in which the different methods employed effected a clean separation of the different proteins. The most convincing account is that given by Hunter [1905], whose paper may be consulted for the earlier literature. Hunter found that each of the serum proteins, euglobulin, pseudoglobulin, and albumin, was capable of exciting precipitin formation, and that the precipitin obtained in each case reacted most strongly with the protein used in its production, but in a modified degree with the others also; in other words, each precipitin showed a relative, or quantitative, but not an absolute, or qualitative specificity for its own protein.

Certain points in the behaviour of the serum-proteins as anaphylactic antigens seemed to deserve further investigation. Doerr and Russ's

description of the progressive weakening of the anaphylactic effect in passing from the euglobulin to the albumin fraction, the pseudoglobulin being intermediate in activity, suggests a possibility that euglobulin may be the only protein in serum capable of producing the reaction, and that the weaker reaction of pseudoglobulin may be due to incomplete separation. The point is not without practical significance, as well as theoretical interest. The antibody of immune horse-serum has been shown to be associated with the pseudoglobulin fraction, and the various methods of artificial concentration have aimed at eliminating the albumin and the euglobulin; but the details of the processes in actual use would seem to ensure a more thorough exclusion of the former. It is clear, on the other hand, if the euglobulin is the anaphylactically active constituent, and the albumin indifferent in this respect, that the aim of any process for purifying antitoxin should be as thorough a removal of euglobulin as is possible in large-scale working.

Methods.

We have confined our attention to horse-serum, specimens of the three proteins from which were available for use. Those which we used in the majority of the experiments were prepared by one of us for a chemical investigation, the results of which have already been published, together with the details of the methods of preparation [Hartley, 1914]. The euglobulin used was that prepared by Panum's method, and probably made a nearer approach to complete purity from the other proteins than did the specimens of pseudoglobulin and albumin, although these were probably as pure as repeated salting-out could make them. An additional specimen of the pseudoglobulin of horse-serum was kindly placed at our disposal by Dr H. Chick, who, after the usual separation by ammonium sulphate, had further purified it by continuous dialysing, with removal of the euglobulin thrown out of solution, during some months. Both the samples of pseudoglobulin were "euglobulin-free," in the sense that the ordinary methods of detection and separation would fail to discover euglobulin in them. But the quantity of a protein needed for sensitisation of the guinea-pig has been shown to be so minute, that it cannot be safely assumed that either was free from such traces of euglobulin as might be perceptibly effective in this direction. The same may be said with regard to a possible contamination of the albumin with minimal remnants of pseudoglobulin. In addition to the specimen above mentioned, a sample of albumin prepared in a different way was used

in several experiments. This was a crystalline albumin prepared by Hopkins and Pinkus's [1898] method, and recrystallised three times, only that fraction being taken of each crop which consisted wholly of large well-formed crystals. This was dialysed free from ammonium sulphate and dried to a scale in vacuo. A portion of it was, for another purpose, dissolved in water, precipitated with cold acetone and ether, extracted for three days with warm ether, and finally taken up in water and recrystallised by Hopkins' method, yielding particularly well-formed crystals. These were again dialysed, scaled, and kept dry. Neither of these preparations showed any notable difference in action from the albumin-fraction obtained by salting-out in the ordinary way.

Most of our experiments were made on guinea-pigs, which were sensitised by hypodermic injection of a small dose, either of whole fresh serum, or of a solution of one of the separated protein preparations. After an interval, varying from 12 up to 31 days, the sensitiveness of the uterine plain muscle to two or more of the separated proteins was tested by the method previously described by one of us [Dale, 1912]. The use of this method has the definite advantage, over experiments made on a series of similarly prepared animals, that no allowance need be made for individual differences of sensitisation. The reaction of one horn of the uterus is tested with one of the pure proteins, and subsequently the reaction of the other horn, which can be regarded with some certainty as equivalent in specific sensitiveness and physiological condition to the first, is tested similarly with another of the pure proteins. Moreover the degree, to which an effective dose of either protein affects the subsequent reaction to the other, can be readily ascertained. By a careful choice of the order in which the doses are given, as much information can often be obtained from one such experiment, with regard to the relative sensitiveness to the different proteins, as would require the injection and reinjection of a long series of animals, if the more usual method of studying anaphylaxis in the guinea-pig were adopted. For purposes of control a few experiments were made by the ordinary method of intravenous reinjection into the intact animal, with observation of the degree of severity of the resulting symptoms as an index of the sensitisation. Young virgin female guinea-pigs were chosen, weighing about 180 g. at the time of the preparatory injection, so that the test of sensitisation, after an interval of two to three weeks, or more, was made when the animals weighed about 250 g. on an average.

The apparatus used was arranged, in all material particulars, similarly to that described by Dale and Laidlaw [1912]. The vessel in which the uterine

horn under experiment was suspended contained, in all cases, 70 cc. of Ringer's solution. The "control" uterine horn was kept, till required, in a tube of the same solution, immersed in the same constant-temperature bath as the experimental vessel. The contents of the experimental vessel, and of the tube containing the control, were oxygenated and stirred by a stream of bubbles. The temperature employed was between 37° and 39° C. The lever recording the uterine contractions magnified twice or thrice, according to the size and activity of the uterus under observation. Similarly the weighting of the lever was varied so as to secure, in each case, efficient extension, without imposing undue resistance to contraction.

The Ringer's solution used was made up according to one or other of the formulae mentioned by one of us in an earlier paper. The results obtained were of the same kind with either, but we gathered the impression that, for keeping the general sensitiveness of the control horn at the same level as that first tested, during a long experiment lasting some hours, the solution called "Ringer I" in the earlier paper was, on the whole, the better. The various proteins tested were made up in Ringer's solution, usually in 1 % solution. The albumin and pseudoglobulin could be dissolved directly in the Ringer's solution without difficulty. Euglobulin so treated was imperfectly soluble. Although the imperfect, opalescent suspension, obtained with Ringer's solution alone, seemed to act perfectly well on the plain muscle when added to the bath, it was considered better, after the first few experiments, to use it in a more highly dispersed condition, in order to obtain a more accurate comparison of its effect with that of pseudoglobulin. With this object, the required amount was weighed out and rubbed up with a little Ringer's solution into a fine emulsion. To this was added 0·1 cc. N/10 NaOH for each 100 mgm. of euglobulin. The relatively clear solution so obtained was then diluted with Ringer's solution to a strength of 1 % euglobulin; the proportion of added soda, after such dilution, corresponding, therefore, to 1/1000 normal. Controls showed that a pure solution of soda, in this strength, when added to the bath in volume corresponding to those of the test doses of euglobulin solution, produced no noteworthy effect on the uterine activity.

A few experiments were also made on anaesthetised dogs, with record of the blood-pressure, to confirm the general applicability of some of the results obtained with guinea-pigs.

Results.

Animals sensitised to whole horse-serum.

We first tested the reaction, to the pure serum proteins, of uterine muscle from guinea-pigs sensitised by a hypodermic injection of 0·01 or 0·1 cc. of whole horse-serum, given two to three weeks previously.

(*a*) *Euglobulin and Pseudoglobulin.* In two cases, out of some eighteen guinea-pigs tested, sensitisation, for some unexplained reason failed, so that the uterine muscle responded but weakly even to large doses of horse-serum. Except in these instances the plain muscle of guinea-pigs, which had received a sensitising injection of whole horse-serum, exhibited a pronounced sensitiveness to the euglobulin and pseudoglobulin of horse-serum. The sensitiveness to the two showed a general parallelism, so that, if one horn of the uterus gave a large contraction in response to 1 mgm. of euglobulin, the second horn usually reacted similarly to 1 mgm. of pseudoglobulin. Similarly, if the first horn tested gave only a small reaction with 1 mgm. of euglobulin, and a subsequent maximal reaction with 10 mgms. of the same, it was usually found that the second horn gave effects of the same order with the same doses of pseudoglobulin.

At first sight, therefore, the results might easily have been interpreted as signifying that the euglobulin and pseudoglobulin were acting as one common antigen. It will be shown later that the effects of sensitising by the separate proteins, instead of by whole serum, exclude such a supposition; but there were clear indications of the distinct antigenic properties of the two globulins, even in the experiments with whole serum. Such indications were obtained by studying the effects of desensitisation. In an earlier paper it was shown that the application of an adequate, effective dose of an antigen, to which the plain muscle has been made anaphylactic, will permanently remove its sensitiveness to that antigen, while leaving it, in other respects, practically normal. The question therefore arises, whether a dose of euglobulin, which renders indifferent to further euglobulin a preparation originally sensitive to both globulins, will at the same time remove its initial sensitiveness to pseudoglobulin, and *vice versâ*. There are three possibilities, and we have found examples of each, in our series of guinea-pigs sensitised by whole serum.

(i) The two globulins may appear to act as a common antigen, so that one horn may respond by a maximal contraction to a certain dose of euglobulin, and be thereafter insensitive not only to euglobulin, but to pseudoglobulin

in like dosage; while the second horn may show an exactly parallel response to, and complete desensitisation by a like dose of pseudoglobulin. This type of reaction is illustrated by the following experimental record, Figs. 1 and 2 reproducing the corresponding tracings.

Guinea-pig No. 9. Sensitised by hypodermic injection of 0·1 cc. of whole horse-serum 16 days previously. Bath-volume 70 cc. Ringer's solution changed after the effect of each dose was complete.

1*st horn of uterus* (Fig. 1).

1 mgm. of euglobulin. Moderate contraction.
10 mgm. of euglobulin. Maximal contraction.
10 mgm. of pseudoglobulin. Nil.

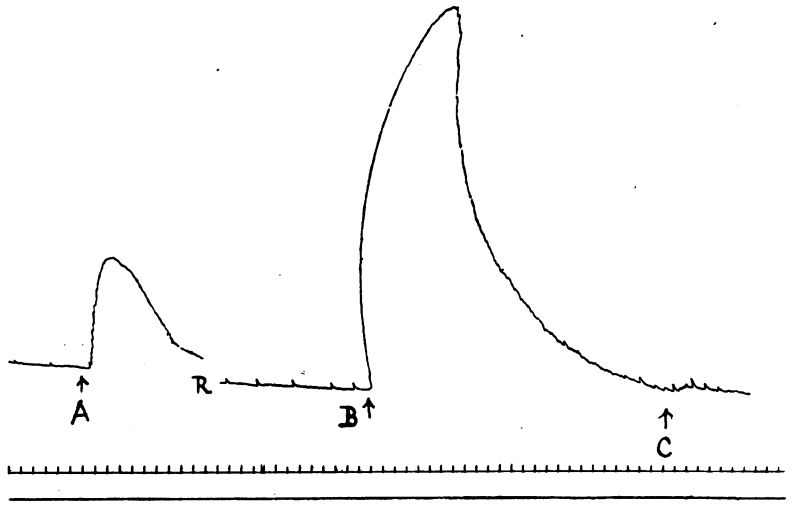

Fig. 1. Guinea-pig sensitised with 0·1 cc. of whole horse-serum. 16th day. 1st horn A. 1 mgm. of euglobulin. B. 10 mgm. of euglobulin. C. 10 mgm. of pseudoglobulin. Time marker (in all figures) marks intervals of 30 seconds. R (in all figures) indicates change of Ringer's solution.

2*nd horn of uterus* (Fig. 2).

1 mgm. of euglobulin. Moderate contraction.
10 mgm. of pseudoglobulin. Maximal contraction.
10 mgm. of euglobulin. Nil.

It will be seen that a dose of 10 mgm. (1 in 7000) of either euglobulin or pseudoglobulin produced a maximal response of the muscle, after the sub-maximal effect of 1 mgm. of euglobulin had been obtained; but that the maximal response, to 10 mgm. of either, left the organ insensitive to the same dose of the other. Such a result is not uncommon, and seems to be

especially frequent in cases, such as that quoted, in which the sensitiveness is not of a high order. It may be noted that the apparently rather smaller amplitude of contraction in response to 10 mgm. of pseudoglobulin (Fig. 2) as compared with that to 10 mgm. of euglobulin (Fig. 1), is attributable to the use of Ringer II in the former, and Ringer I in the latter case, for the purpose of comparing their suitability for this type of experiment. It is no index, therefore, of preferential sensitisation, which is excluded by the perfect mutual desensitisation.

(ii) There may be sensitisation, to either euglobulin or pseudoglobulin in higher degree than to the other. The difference was in no case very conspicuous, and its demonstration depended chiefly on the phenomena of desensitisation. Thus we might find that one horn responded maximally to

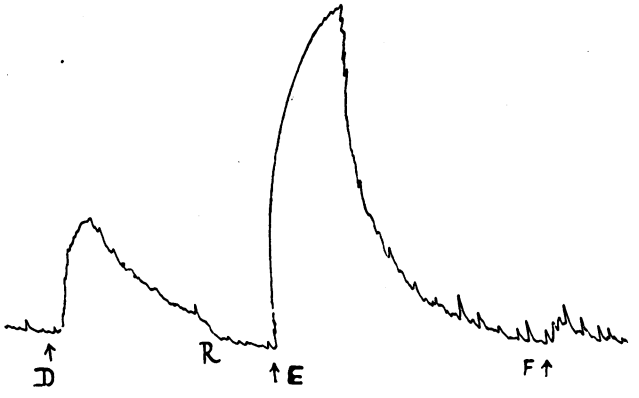

Fig. 2. From same experiment as Fig. 1. 2nd horn. D. 1 mgm. of euglobulin. E. 10 mgm. of pseudoglobulin. F. 10 mgm. of euglobulin.

1 mgm. of euglobulin, gave a second smaller response to 10 mgm. of euglobulin, and was thereafter desensitised not only to euglobulin, but to pseudoglobulin also; while the second horn after practically similar responses to 1 mgm. and 10 mgm. of pseudoglobulin, would be completely insensitive to this protein, but still capable of giving a large response to 10 mgm. of euglobulin. In another case the reverse condition would obtain, the plain muscle desensitised to pseudoglobulin failing to respond to euglobulin, while the second horn, when desensitised to euglobulin, still responded to pseudoglobulin. A couple of examples will suffice to illustrate the point, the importance of which was that it gave us the first indication of an antigenic distinction between the two proteins, which experiments on sensitisation with the separate proteins confirmed.

Experiment 4. Guinea-pig sensitised with 0·1 cc. of horse-serum. 22nd day.

1*st horn.*

1 mgm. euglobulin. Maximal contraction.
5 mgm. euglobulin. Smaller contraction.
5 mgm. euglobulin. Nil.
5 mgm. pseudoglobulin. Nil.

2*nd horn.*

1 mgm. pseudoglobulin. Maximal contraction.
5 mgm. pseudoglobulin. Nil.
5 mgm. pseudoglobulin. Nil.
5 mgm. euglobulin. Large contraction.
5 mgm. euglobulin. Nil.

Experiment 7. Guinea-pig sensitised with 0·1 cc. horse-serum. 21st day.

1*st horn.*

1 mgm. pseudoglobulin. Good contraction.
1 mgm. pseudoglobulin. Nil.
10 mgm. pseudoglobulin. Small contraction.
10 mgm. pseudoglobulin. Nil.
10 mgm. pseudoglobulin. Nil.
10 mgm. euglobulin. Maximal contraction.

2*nd horn.*

1 mgm. euglobulin. Large contraction (? Maximal).
1 mgm. euglobulin. Nil.
10 mgm. euglobulin. Small contraction.
10 mgm. euglobulin. Nil.
10 mgm. euglobulin. Nil.
10 mgm. pseudoglobulin. Nil.
40 mgm. pseudoglobulin. Nil.

The above two records show preferential sensitisation to euglobulin. In the following the predominant sensitiveness to pseudoglobulin is shown not only in the greater effect of desensitisation to this protein on the subsequent response to euglobulin, but in the lower initial effective dose of the former.

Guinea-pig sensitised with 0·04 cc. antidiphtheritic horse-serum + 1 test dose diphtheria toxin. 24th day.

1*st horn.*

1 mgm. pseudoglobulin. Full contraction.
10 mgm. pseudoglobulin. Small contraction.
10 mgm. pseudoglobulin. Nil.
10 mgm. euglobulin. Slight contraction.

2*nd horn.*

1 mgm. euglobulin. Nil.
10 mgm. euglobulin. Good contraction.
10 mgm. euglobulin. Moderate contraction.
10 mgm. euglobulin. Small contraction.
10 mgm. euglobulin. Very small contraction.
10 mgm. pseudoglobulin. Large contraction.
10 mgm. pseudoglobulin. Nil.

In this record there is an obvious indication that complete desensitisation to euglobulin is more difficult to achieve than that to pseudoglobulin. This point is clearly discernible in a number of our records. A probably related effect is also to be seen in the above, namely, the tendency of the plain muscle to recover from the desensitisation to euglobulin, and show a revival of response to this protein at a later stage of the experiment. This may indicate a tendency of the euglobulin-antibody complex to dissociate, or it may be attributable to the physical properties of the euglobulin solution, on account of which this antigen less readily reaches and saturates the antibody in the deeper layers of the uterine muscle. We have no direct evidence in favour of either interpretation, but the fact is repeatedly illustrated in our records.

(iii) In some of our records from guinea-pigs sensitised with whole horse-serum, the distinct antigenic properties of the two globulins are made evident by the fact that, while the plain muscle responds with equal readiness to an initial dose of either eu- or pseudoglobulin, desensitisation to either of these leaves a remainder of sensitiveness to the other. This is most readily shown by producing a *relative* desensitisation, by using small doses throughout, as in the following record.

Experiment 12. Guinea-pig sensitised with 0·1 cc. horse-serum. 14th day.

1*st horn.*

1 mgm. euglobulin. Good contraction.
1 mgm. euglobulin. Small contraction.

1 mgm. euglobulin. Nil.
1 mgm. pseudoglobulin. Fair contraction.
1 mgm. pseudoglobulin. Nil.

2nd horn.

1 mgm. pseudoglobulin. Good contraction.
1 mgm. pseudoglobulin. Nil.
1 mgm. euglobulin. Fair contraction.

It is probable, to judge from the results of other experiments, that the 1st horn, when no longer giving a perceptible response to 1 mgm. of euglobulin, would have responded to 10 mgm.; and similarly with pseudoglobulin in the case of the second horn. The point to be emphasised is that, in either case, the muscle, when reduced to a condition in which it fails to respond at all to 1 mgm. of one of the globulins, still shows a definite sensitiveness to the same dose of the other.

(*b*) *Albumin.* Our earlier results seemed to support the finding of Doerr and Russ, that serum albumin did not act as an anaphylactic antigen. In a series of preparations from guinea-pigs sensitised to whole serum, we found that the plain muscle seemed completely indifferent to a dose of 5 or 10 mgm. of the albumin, subsequently giving a maximal response to 1 mgm. of either globulin. The result seemed so definite, and was obtained with such regularity in the early series, that for some time we abandoned experiments with albumin, considering its inefficacy as settled. At a later stage of the investigation six guinea pigs received the usual sensitising injection of 0·1 cc. of horse-serum, to test another point, and it happened that the uterus was tested with 1 mgm. of pure serum albumin, which produced a typical, nearly maximal, response. The uteri of the other guinea-pigs of this series were then tested with albumin, and all showed a similar high degree of sensitiveness to this protein. The difference could not be attributed to a deficiently purified albumin, since the preparation which now proved an effective stimulus was the same as that which had previously proved ineffective; and the highly purified sample obtained by recrystallising four times, as described above, was quite as effective a stimulant as that purified by the precipitation method only. The only difference between the two series was in the time elapsing between the sensitising injection and the performance of the test. In the earlier series, in which no sensitiveness to albumin was detected, the interval was two to three weeks; in the later series, in which a good reaction to albumin was

observed, it was upwards of thirty days—four to five weeks. A further series of six guinea-pigs was therefore prepared, being sensitised with 0·1 cc. horse-serum and kept for twenty-nine to thirty days before testing, with the same result, that after this interval a sensitiveness of the plain muscle to albumin had developed.

There was some indication that, concurrently with the late development of the sensitiveness to albumin, the sensitiveness of the plain muscle to the globulins, which attains its maximum somewhere between the 14th and 21st days, undergoes some decline, to judge by the size of the initial dose needed to produce a pronounced reaction. The following record illustrates the point.

Fig. 3. Guinea-pig sensitised with 0·1 cc. of whole horse-serum. 31st day. 1st horn. A. 1 mgm. of albumin.

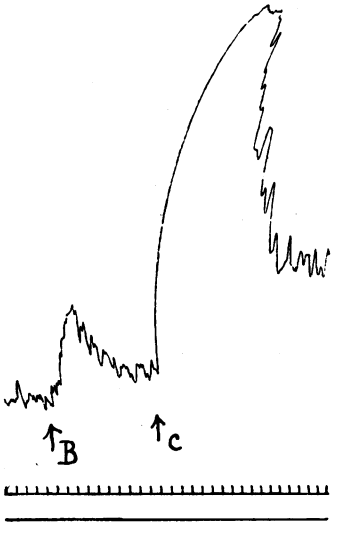

Fig. 4. Same experiment as Fig. 3. 2nd horn. B. 1 mgm. of euglobulin. C. 10 mgm. of euglobulin.

Experiment 26. Guinea-pig sensitised with 0·1 cc. horse-serum. 31st day.

1*st horn.*

1 mgm. albumin.	Maximal contraction (see Fig. 3).
10 mgm. albumin.	Weak contraction.
10 mgm. albumin.	Nil.
1 mgm. euglobulin.	Nil.
10 mgm. euglobulin.	Moderate contraction.

2nd horn.

1 mgm. euglobulin. Small contraction.
10 mgm. euglobulin. Maximal contraction (see Fig. 4).
10 mgm. euglobulin. Nil.
10 mgm. albumin. Nil.

It will be noticed that though the plain muscle, in this case, showed a higher sensitiveness to albumin than to euglobulin, as judged by its response to a small dose (1 mgm.) of either, it is more readily desensitised to albumin by an effective dose of euglobulin, than to euglobulin by albumin.

The results obtained, by observing the response of isolated plain muscle, suggested that the failure of previous workers to observe an anaphylactic reaction in the serum-sensitive guinea-pig, when pure albumin was used for the reinjection, might have been due to the allowance of an insufficient incubation-period for the development of sensitiveness to this protein. It seemed desirable, therefore, to confirm, by experiments on the intact animal, the fact that true anaphylaxis to serum albumin does occur, though late in its development; in other words, to ensure that the reaction of the isolated plain muscle, *in vitro*, gives, in this case also, a genuine indication of the occurrence of the anaphylactic condition, as seen in the whole animal. A guinea-pig from the same series as that used in Experiment 26, and similarly sensitised by a hypodermic injection of 0·1 cc. of horse-serum, was therefore tested on the 32nd day after sensitisation. The albumin was made up for the reinjection in 1 % solution in physiological saline. Of this solution 1 cc. (10 mgm. of albumin) was injected into the jugular vein, exposed by a small incision under local cocaine-anaesthesia. Symptoms appeared immediately after the completion of the injection, and typical anaphylactic death followed in about 3 minutes. The post-mortem appearances were quite characteristic—fixed distension of the lungs, and retarded coagulation of the blood.

It seemed desirable to confirm, in another species of animal, this efficacy of serum-albumin as an anaphylactic antigen, provided the incubation period is sufficiently long. A dog therefore received a sensitising injection of 5 cc. of horse-serum hypodermically. On the 28th day after this injection the dog was anaesthetised by A.C.E. mixture, after a preliminary injection of morphia. Arrangements were made for recording the blood-pressure from the carotid artery, and injection into the femoral vein. The record was started, and 20 cc. of a 5 % solution in saline of the pure albumin of horse-serum were then run slowly into the vein. A profound and typical shock

occurred, the blood-pressure commencing to fall rapidly during the injection, continuing to fall after the injection was completed, and remaining long at a very low level. The fall of pressure was accompanied by the typical dyspnoea, and a sample of blood, obtained while the depression persisted from the other carotid artery, showed no sign of clotting after standing for 24 hours.

Guinea-pigs sensitised to individual proteins.

(*a*) *Sensitised to euglobulin.* Sensitisation with euglobulin produced a more clearly specific sensitiveness than that with either of the other proteins. When a guinea-pig was killed 13 to 17 days after a preliminary injection of 1 mgm. of euglobulin, its uterine plain muscle responded typically to 1 mgm. or 10 mgm. of euglobulin, but was apparently indifferent either to albumin or pseudoglobulin, at any rate within the limits of dosage employed in these experiments (see Figs. 5 and 6).

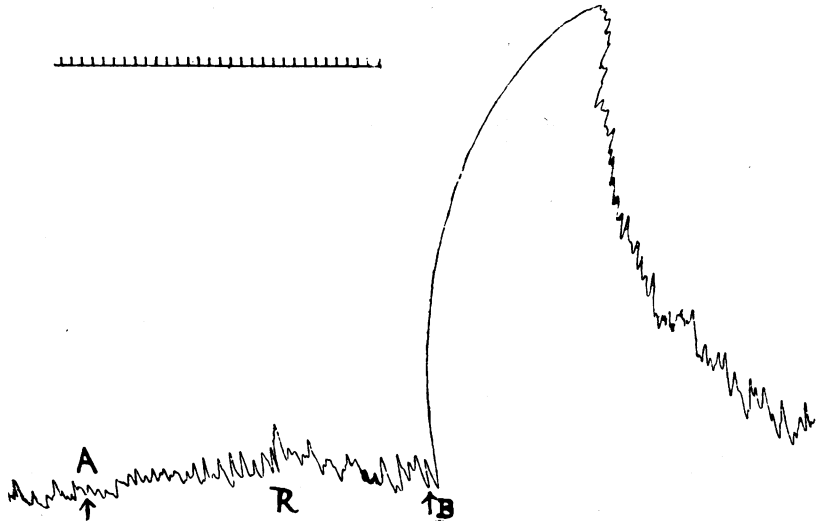

Fig. 5. Guinea-pig sensitised with 1 mgm. of euglobulin. 17th day. A. 5 mgm. of albumin. B. 1 mgm. of euglobulin.

(*b*) *Sensitised to pseudoglobulin.* The available samples of pseudoglobulin produced a sensitisation to either pseudoglobulin or euglobulin, but the sensitiveness to the former was always definitely superior, as the following record shows.

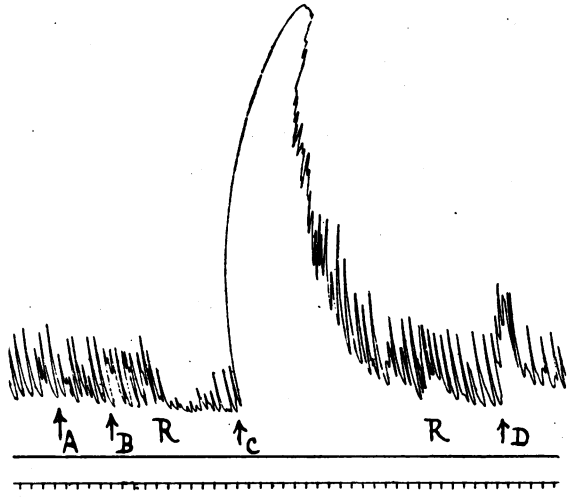

Fig. 6. Guinea-pig sensitised with 1 mgm. of euglobulin. 13th day. A. 1 mgm. of pseudoglobulin. B. 10 mgm. of pseudoglobulin. C. 10 mgm. of euglobulin. D. 10 mgm. of euglobulin.

Experiment 30. (See Figs. 7, 8 and 9.) Guinea-pig sensitised with 1 mgm. of pseudoglobulin. 22nd day.

1*st horn.*

1 mgm. pseudoglobulin. Maximal contraction.

2*nd horn.*

1 mgm. euglobulin. Moderate contraction.
10 mgm. euglobulin. Good but submaximal contraction.
10 mgm. euglobulin. Small contraction.
10 mgm. euglobulin. Nil.
10 mgm. pseudoglobulin. Good but submaximal contraction.

It is clear that 1 mgm. pseudoglobulin, as a first dose, is much more effective than 1 mgm. euglobulin under the same conditions; further that complete desensitisation to euglobulin weakens, but by no means annuls the sensitiveness to pseudoglobulin.

Experiment 35. Guinea-pig sensitised with 0·1 mgm. pseudoglobulin 18 days.

1*st horn.*

0·1 mgm. pseudoglobulin. Small contraction.
1 mgm. pseudoglobulin. Moderate contraction.
1 mgm. pseudoglobulin. Nil.
10 mgm. euglobulin. Very small contraction.

2nd horn.

1 mgm. euglobulin. Nil.
10 mgm. euglobulin. Maximal contraction.
10 mgm. euglobulin. Nil.
1 mgm. pseudoglobulin. Small contraction.

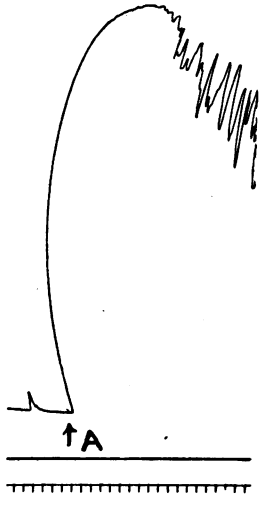

Fig. 7. Guinea-pig sensitised with 1 mgm. of pseudoglobulin. 22nd day. 1st horn. A. 1 mgm. of pseudoglobulin.

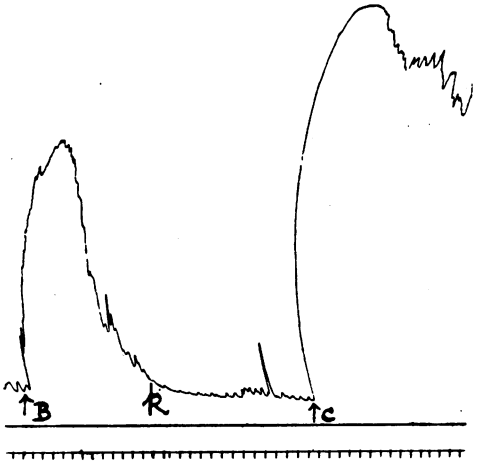

Fig. 8. Same experiment as Fig. 7. 2nd horn. B. 1 mgm. of euglobulin. C. 10 mgm. of euglobulin.

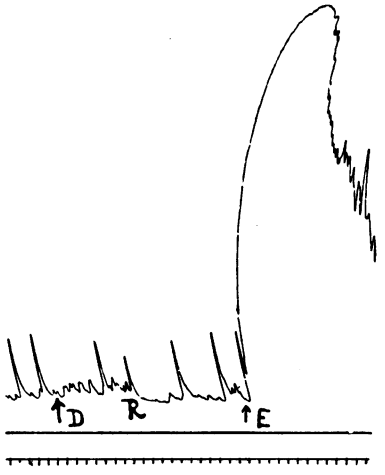

Fig. 9. Continuation of Fig. 8. D. 10 mgm. of euglobulin. E. 10 mgm. of pseudoglobulin.

In this experiment an error was made in dilution, which resulted in the dose of pseudoglobulin being throughout only one-tenth of what had been intended. The mistake, however, was not without advantage, in bringing out very clearly the preponderance of sensitiveness to pseudoglobulin. For the result showed that desensitisation to 1 mgm. of pseudoglobulin almost abolished the response to 10 mgm. of euglobulin; while after desensitisation to 10 mgm. of euglobulin, the muscle responded definitely, though not powerfully, to 1 mgm. of pseudoglobulin. It may also be noted that, whereas the first horn gave a small, but quite definite response even to 0·1 mgm. pseudoglobulin—*i.e.* to a dilution in the testing-bath of 1 in 700,000—the second horn gave no response at all to 1 mgm. (1 in 70,000) of euglobulin, but was stimulated to maximal contraction by 10 mgm. (1 in 7,000).

Sensitised to serum albumin.

All the guinea-pigs used in these tests received a sensitising hypodermic injection of 1 mgm. of purified serum albumin. Unfortunately lack of time, and the exhaustion of our small stock of purified pseudoglobulin, prevented any discriminating tests between the antigenic properties of pseudoglobulin and albumin, which would have given information of some interest. We were able, however, to show, more definitely than with the guinea-pigs sensitised to whole serum, the antigenic independence of albumin and euglobulin. Few experiments were made with isolated muscle, and it will be sufficient to quote one. It will be noted, both in this case and in the records of experiments on the intact animal, that when pure albumin is used for sensitisation, anaphylaxis to this protein, though still somewhat slowly developed, appears at an earlier stage than when the immunity reaction is complicated by the presence of the other serum proteins in the sensitising inoculum.

Experiment 35. Guinea-pig sensitised with 1 mgm. serum albumin hypodermically. 22nd day.

1st horn.

1 mgm. serum albumin. Good, but not maximal contraction.
10 mgm. serum albumin. Nil.
10 mgm. euglobulin. Nil.

2nd horn.

1 mgm. euglobulin. Nil.
10 mgm. euglobulin. Trace of response (doubtfully specific).
1 mgm. albumin. Good, but not maximal contraction.

For the experiments on anaphylaxis *in vivo* two guinea-pigs (*A* and *B*) were first sensitised, each with 1 mgm. albumin as usual. Twenty-two days later, when each weighed 250 g. *A* was tested by intravenous injection of 10 mgm. albumin in 1 cc. of saline. This caused pronounced symptoms—coughing, chattering, erection of hair, falling on the side. After about 20 minutes, however, the animal was obviously recovering, and 10 minutes later was practically normal again. Next day it was perfectly well.

On the 35th day both guinea-pigs weighed about 275 g. *B* received 5 mgm. of albumin in 0·5 cc. of saline intravenously, showed immediate pronounced symptoms, and died within 2 minutes.

A, which had survived the injection on the 22nd day, was given a second injection of 10 mgm. on the 35th day. Symptoms developed slightly more slowly than in *B*, but death occurred within 3 minutes.

Both animals showed the typical post-mortem picture of acute anaphylaxis—lungs fixed in distension, slowly clotting blood, etc.

A further series of twelve guinea-pigs (numbered I–XII) were sensitised with 1 mgm. of albumin and examined as follows.

No. of guinea-pig	Weight in g.	No. of days after sensitisation	Dose given intravenously	Result
I	275	10	Albumin 20 mgm.	Slight symptoms, recovered
II	275	10	„ 10 „	Nil
III	275	17	„ 20 „	Death in 3 mins.
IV	250	17	„ 10 „	„ 4 „
V	250	17	„ 5 „	„ 5 „
VI	225	17	„ 2 „	„ 3 „
VII	215	17	„ 1 „	„ 3 „
X	220	27	Euglobulin 5 mgm.	Nil
XII	210	27	Albumin 0·5 mgm.	Death in 2 mins.
X (2nd injection)	220	28	„ 1 mgm.	„ 4 „
I (2nd injection)	300	30	Albumin 10 mgm.	Nil
II (2nd injection)	300	30	„ 1 „	Nil

Guinea-pig VIII was used for the isolated muscle test quoted above; guinea-pigs IX and XI died from accidental causes before testing.

There are several points worth noting in the above table. The sensitiveness to albumin is practically non-existent on the 10th day, by which sensitiveness to whole serum is well developed in animals previously injected with this. By the 17th day, however, in this series a very high sensitiveness to albumin has appeared, the lower limit of fatal dosage for guinea-pigs of this size not being reached in the experiment. The experiments on the 27th and 28th days show that there is no reaction to euglobulin in these

guinea-pigs, even in a dose at least ten times as large as the fatal dose of albumin; nor does the injection of euglobulin notably modify the effect of albumin given on the next day. Lastly, it is seen that, when large injections have been given on the 10th day, *i.e.* before sensitiveness to albumin has appeared, no sensitiveness of the animal to albumin can be detected by intravenous injections given on the 30th day. It has been shown by one of us that such lack of apparent sensitiveness may be due either to genuine desensitisation of the tissues, or to an excess of antibody in the circulating fluids, protecting from the antigen tissues which, when isolated, are highly sensitive. It must remain an open question, for the present, which of these two conditions accounted for the lack of intoxication in this instance.

Note on Anaphylaxis to Pure Egg-albumins.

The production in the guinea-pig of a high degree of anaphylaxis to the pure, crystallised albumin of the hen's egg has been previously described by Armit [1910] and by Wells [1908]. Wells [1911] has also shown that ovo-vitellin, from egg-yolk, acted as a quite separate antigen from the proteins of egg-white[1]. In the preceding sections we have given evidence of the separate action, as anaphylactic antigens, of the different proteins of one serum. By way of contrast it seems appropriate to make preliminary mention here of some experiments made by one of us (H. H. D.) on anaphylaxis to the pure crystalline albumins from the egg-white of hens and ducks, in the preparation of which Dr A. J. Ewins kindly co-operated. The albumins were crystallised by the familiar method of Hopkins [1899], three successive crystallisations being given to each sample. The eggs were obtained from a private source, and there can be no question of any mistake in specific identification. The albumin from the ducks' eggs was rather more difficult to crystallise than that from hens, a larger addition of acid being required to initiate the first crystallisation. When once obtained in clean crystals, however, it was recrystallised as readily as that of the hen. The final crop of crystals, in both cases, was dialysed free from ammonium sulphate, and the watery solutions were evaporated to dry scale-preparations at room-temperature *in vacuo*, solutions being then prepared as required. Female

[1] There is a somewhat extensive literature on the crossed specificity of different egg-whites, which has not been dealt with here, since the experiments made by us at present have been concerned solely with the crystalline albumins.

guinea-pigs received injections of 0·1–1 mgm. of either albumin, and the isolated uterine plain muscle was tested for sensitiveness after three weeks. The result can be stated in a line. There was no evidence of any antigenic distinction between the two albumins. The plain muscle of guinea-pigs, which had been injected with hen's albumin was quite as sensitive to duck's albumin as to hen's, and the same was true of preparations from guinea-pigs injected with duck's albumin. Moreover an effective dose of either albumin

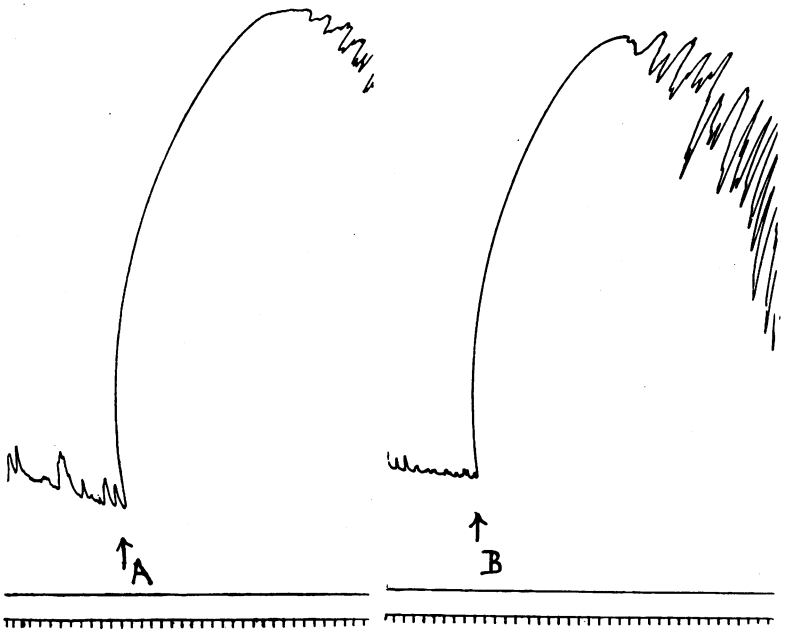

Fig. 10. Guinea-pig sensitised with 1 mgm. of crystalline albumin of hen's egg-white. 22nd day. A. 1st horn. Effect of 0·05 mgm. duck's egg-albumin. B. 2nd horn. Effect of 0·05 mgm. hen's egg-albumin.

was followed by complete insensitiveness to the other, irrespective of which had been used as the sensitising dose. If the notebook record had been lost, it would have been impossible, from the response of the plain muscle, to form any opinion as to which albumin had been used for sensitisation. Fig. 10 illustrates the corresponding reactions of the two uterine horns from a guinea-pig sensitised with hen's albumin. It may be added that neither preparation had any effect on uterine muscle from normal, unsensitised guinea-pigs.

Summary and Discussion.

The following are the main conclusions to be drawn from the above experiments:

1. Each of the three proteins separable from horse-serum by their physical and chemical properties—euglobulin, pseudoglobulin, albumin—can act as an anaphylactic antigen.

2. A guinea-pig which has received a sensitising injection of one of these proteins is more sensitive to that one than to either of the others from the same serum. In some cases the sensitisation seems to be rigidly "specific," as when euglobulin produces sensitiveness to euglobulin but not to pseudoglobulin, or when albumin produces a high degree of sensitiveness to albumin but none to euglobulin.

3. The sensitiveness of the guinea-pig to albumin is later in development than that to the globulins; this difference possibly accounts for the fact that previous authors failed to detect the sensitising property of albumin. The difference is especially marked when sensitisation is carried out with whole serum, containing all three.

4. An effective dose of any of the proteins, to which the guinea-pig's plain muscle has been sensitised, partially or completely desensitises it to the other proteins of the same serum.

5. The crystalline albumins from the white of the eggs of hens and ducks show no distinction of antigenic properties.

The effects of "crossed" desensitisation, in particular, may give the impression that there is a considerable community of antigenic function among the proteins of one serum. Such an impression, however, is not completely justified, since one of us (H. H. D.) has previously shown that, when a guinea-pig is simultaneously sensitised to two or more antigens of widely different origin, and of undoubted antigenic independence—*e.g.* egg-white and horse-serum—desensitisation of the plain muscle to one may greatly weaken the sensitiveness to the other. On the other hand, the fact that sensitisation with one of the globulins frequently produces a smaller, but quite definite sensitiveness to the other, shows that, at any rate, the preparations of these two substances at our disposal were not completely independent in their antigenic properties. But the question necessarily arises whether this overlap is a genuine phenomenon, or is due to the fact that, even in such elaborately purified preparations as those which we used, the separation was

not ideally perfect. This possibility is worthy of consideration, in that the amount of a protein needed to evoke some degree of sensitiveness is almost inconceivably small. Wells [1908], for example, succeeded in sensitising guinea-pigs with 1/20,000 milligram of egg-albumin. This explanation of the overlap in sensitisation is favoured by the evidence that it appears most prominently when the impurity of the sensitising protein is most likely to be considerable. The purification of euglobulin is facilitated by its insolubility in weakly acidulated water. We have found that a rigidly specific sensitiveness to euglobulin occurs comparatively frequently. On the other hand, we never succeeded, by injection of pseudoglobulin in producing a sensitiveness to pseudoglobulin alone; sensitiveness to euglobulin in addition was always detectable, though it might be relatively weak, especially when the sensitising dose was small. But the purification of pseudoglobulin from the last traces of euglobulin is so difficult, that it may be doubted whether it is possible by existing methods. It is similarly difficult to make a sharp separation between pseudoglobulin and albumin, and we think it probable that a similar overlap of sensitisation would have been detected between these two proteins. Unfortunately the material was exhausted before this point had been put to the test. Again, if we regard the overlap of antigenic properties as due to incomplete separation, it would be expected that the sharpest distinction would be observed between euglobulin and albumin, each of which can be obtained with tolerable certainty uncontaminated by the other. The results were quite in accordance with this expectation, since the plain muscle from a guinea-pig sensitised with euglobulin seemed quite indifferent to albumin, and *vice versâ*. On the whole, then, the evidence seems to us in favour of the view that the different proteins of a serum, in so far as they can be obtained pure from one another, act as independent anaphylactic antigens.

This narrowing down of immunological "specificity," to a discrimination between the different pure proteins of a single tissue of one species, is by no means new. Wells [1911], as above mentioned, found evidence of complete antigenic independence between the "ovovitellin" of egg-yolk, on the one hand, and the proteins of egg-white on the other; he found, however, a considerable overlap in the antigenic properties of the albumin and globulin of egg-white, however carefully they were purified. Ovomucoid was again an independent antigen. Wells and Osborne [1911, 1913] have furnished numerous instances in which the different pure proteins from one plant species act as separate antigens, while the corresponding pure proteins from allied species have common antigenic properties. Such evidence gives to the

fundamental problem of immunity a more hopeful aspect, by its suggestion that the antigenic distinction is possibly dependent on such properties of the different proteins as may be open to the ordinary methods of chemical and physical enquiry, and not necessarily on some biological difference of so mysterious a nature, as to elude investigation by such methods. Our own evidence of the antigenic independence of the proteins from one blood-serum, and of the community of antigenic function between the pure albumins of egg-white from species so widely separated as the fowl and the duck, has the same tendency. It seems clear to us that every sensitisation with a whole serum, for example, is in reality a complex multisensitisation; and that it is essential to work with individual pure proteins, in any attempt to investigate the nature of the difference, of which antigenic independence is an expression.

The question has often been debated whether the difference is a chemical or a physical one, without giving these terms any greater precision of application. One possibility of chemical difference we are in a position to eliminate. An analysis of the separated proteins from horse-serum, used by us in these experiments, had been carried out by one of us [P. Hartley, 1914] by the aid of van Slyke's method. It was found that albumin, as others had shown, differed from the globulins in yielding, on hydrolysis, a higher proportion of the diamino-acids, and in containing a higher proportion of cystine. It was further shown that, of the diamino-acids, the excess yielded by albumin, as compared with the globulins, was greatest in the case of lysine, which albumin contained in about a 50 % higher proportion. No such difference was found between the two globulins, which, as regards the distribution of their nitrogen among the different amino-acids, showed no difference of any note. There is no help to be obtained, therefore, in this direction towards an explanation of the antigenic difference between the globulins. It is clear, however, that there is abundant room for difference of chemical structure even between protein molecules built up from the same amino-acids in identical proportions. Indeed, if the highly specific combining properties, which give a protein molecule its antigenic character, are related to chemical structure at all, it seems likely that they would be as much dependent on the pattern of the structure of the molecule, on the order in which the amino-acids are linked up in it, as on their nature and the proportions in which they are present. A relation between the stereo-chemical and antigenic characters of the protein molecule is, indeed, definitely suggested by the observation of Ten Broek [1914], who found that a protein racemised by Kossel's method had lost all

power of acting as an antigen, just as Dakin and Dudley [1913] found that it had lost its susceptibility to the action of proteolytic enzymes. Dakin [1912] has given reason for believing that the amino-acids which escape racemisation in this process, and are therefore obtained in the natural, optically active condition on subsequent acid hydrolysis of the racemised protein, are those occupying the terminal positions of the peptide chains out of which the protein molecule is built. Dudley and Woodman [1915], using Dakin's method, found evidence of a structural difference of the kind under discussion between the caseinogens of cow's and of sheep's milk—proteins consisting of identical amino-acids in identical proportions. In the case of sheep's caseinogen the tyrosine wholly and the lysine largely escaped racemisation, while they were both completely racemised in that of cow's caseinogen. The instance is an unsatisfactory one for our present purpose, since the antigenic properties of caseinogen are relatively feeble, and the caseinogens from different species show no clear disparity of antigenic properties. In the only other case of proteins with identical amino-acid content which have hitherto been thus compared, the euglobulin and pseudoglobulin of colostrum, there is presumptive evidence of an antigenic difference of the same kind as that which we find existing between the corresponding serum-proteins. In this instance Dudley and Woodman detected no difference by the racemisation method. So that, so far as evidence of this kind has been obtained at present, it fails to support, and might even seem to discredit the view that antigenic properties depend on the structural pattern of the protein molecule. We have an example of structural difference, without antigenic disparity, and another, with no structural difference detected, in which some degree of antigenic independence may be presumed. It must be remembered, however, that the method reveals only one kind of structural difference, the effect of which on the antigenic properties might well be subsidiary to other differences, which the method fails to detect. The observation that two similar proteins from different species, the caseinogens of the cow and sheep, while as yet indistinguishable by other methods, show a definite difference in the order of amino-acid linkage, remains highly significant, and seems to indicate the lines along which the possibility of a connexion between antigenic properties and chemical structure may yet be investigated.

It has not been assumed, in thus considering the possibility of a chemical basis for antigenic specificity, that the combination between antigen and antibody is of the type of an ordinary chemical union. There is, of course, much evidence indicating that the association has the character rather of

an adsorption. This, however, by no means precludes the possibility that it may be conditioned ultimately by the structure of the antigenic molecule. There is similarly strong evidence in favour of regarding the association between an enzyme and its substrate as an adsorption; yet it is essentially dependent on the configuration of the substrate molecule. Again, Barger and his co-workers [1915] have demonstrated the connexion between the formation of coloured adsorption-compounds with iodine and a certain type of structure in organic compounds. They also showed, however, the dependence of the reaction on surface-phenomena, in that a colloidal solution, or suspension of the substance in amorphous aggregates, was necessary for the formation of the typical coloured complex with iodine. It is not improbable that the antigenic behaviour of a protein is similarly conditioned by its state of dispersion. Miss Chick [1914] and others have given reason for considering euglobulin as possibly formed by association of pseudoglobulin with a lipoid, which gives to euglobulin its phosphorus content and its characteristic physical properties. It is conceivable that the antigenic difference between a pseudoglobulin and the euglobulin from the same serum may be due to the peculiar physical characteristics conferred on the euglobulin by associated lipoid. We hope later to test this possibility by comparing the antigenic action of a pseudoglobulin, with that of an artificial "euglobulin" made from it. But even if that were the case, such characteristics could hardly be more than one factor in the phenomenon of specificity; and to form an idea of its significance, it would be necessary to examine the degree of antigenic independence exhibited by pure euglobulins from the sera of different species. Using the purest proteins obtainable as antigenic units, it should eventually be possible to compare different proteins from the same species, and similar proteins from different species, in respect of their molecular configuration and also of their physical properties, and to trace the relation of each type of character to the antigenic individuality of the substances examined.

A word may be added on some practical bearings of our results. Since all the proteins of a serum can act as anaphylactic antigens, it is evident that the ideal to be aimed at, in concentrating the curative element in a specific immune serum, is simply the reduction of the ratio of total protein to antitoxic value; for the purpose of reducing serum-reactions, the elimination of albumin seems to be as important as that of euglobulin, when the pseudoglobulin is the fraction carrying the therapeutic power. Our observation of the relatively long latent period of the sensitiveness to albumin is also suggestive in connexion with the successive crops of serum rash, which have

been recorded in certain patients as the result of one injection of a serum. It has been suggested [Goodall, 1907] that these represent separate reactions to the sera of the different horses which may have contributed to the serum. It seems to us more reasonable to suppose that they represent the successive appearances, at different time intervals, of sensitiveness to the different serum-proteins.

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